MOLECULAR ONCOLOGY 5 (2011) 48–60

available at www.sciencedirect.com

www.elsevier.com/locate/molonc

Genomic instability and copy-number heterogeneity of chromosome 19q, including the kallikrein locus, in ovarian carcinomas

Jane Bayani[a,b], *Paula Marrano*[c], *Cassandra Graham*[a,c], *Yingye Zheng*[d], *Lin Li*[d], *Dionyssios Katsaros*[e], *Heini Lassus*[f], *Ralf Butzow*[f], *Jeremy A. Squire*[g], *Eleftherios P. Diamandis*[a,b,h,*]

[a]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[b]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Joseph and Wolf Lebovic Health Complex, 6th Floor, Room 6-201, Box 32, 60 Murray Street, Toronto, Ontario M5T 3L9, Canada*
[c]*Department of Laboratory Medicine and Pathobiology, Hospital for Sick Children, 555 University Ave., 3rd Floor, Toronto, Ontario M5G 1X8, Canada*
[d]*Department of Biostatistics & Bioinfomatics, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. North, PO Box 19024, Seattle, WA 98109 1024, USA*
[e]*Department of Obstetrics and Gynecology, University of Turin, via Ventimiglia 3, 10126 Torino, Italy*
[f]*Department of Pathology, University of Helsinki, Research Laboratory, Haartmaninkatu 8 FIN-00029 HUS Helsinki, Finland*
[g]*Department of Laboratory Medicine and Pathobiology, Queen's University, Kingston General Hospital, Translational Laboratory Research, NCIC Clinical Trials Group, Room 201e, 88 Stuart St Queen's University, Kingston, Ontario K7L 3N6, Canada*
[h]*Department of Clinical Biochemistry, University Health Network, Toronto, Ontario, Canada*

ARTICLE INFO

Article history:
Received 7 July 2010
Received in revised form
3 August 2010
Accepted 4 August 2010
Available online 11 August 2010

Keywords:
FISH
Copy-number
Chromosomal instability
Structural rearrangement
Kallikrein locus
Ovarian carcinoma

ABSTRACT

Many tissue kallikrein (*KLK*) genes and proteins are candidate diagnostic, prognostic and predictive biomarkers for ovarian cancer (OCa). We previously demonstrated that the *KLK* locus (19q13.3/4) is subject to copy-number gains and structural rearrangements in a pilot study of cell lines and ovarian cancer primary tissues, shown to overexpress KLK gene family members. To determine the overall frequency of genomic instability and copy-number changes, a retrospective study was conducted using formalin-fixed paraffin embedded (FFPE) tissues. Eighty-one chemotherapy naïve serous OCas were examined using 3-colour fluorescence *in situ* hybridization (FISH) to identify structural and numerical changes on 19q, including the *KLK* locus; in addition to immunohistochemistry (IHC) for KLK6, which has been shown to be overexpressed in OCa. The *KLK* locus was subject to copy-number changes in ~83% of cases: net gain in 51%, net loss in 30% and amplified in 2%; and found to be chromosomally unstable ($p < 0.001$). All cases showed a wide range of immuoreactivity for KLK6 by IHC. Although no strong correlation could be found with copy-number, the latter was contributing factor to the observed KLK6 protein overexpression. Moreover, univariate and multivariate analyses showed an association between the net loss of the *KLK* locus and longer disease-free survival. Interestingly, FISH analyses indicated that chromosome 19q was subjected to structural rearrangement in 62% of cases and was significantly

* *Corresponding author*. Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Joseph and Wolf Lebovic Health Complex, 6th Floor, Room 6-201, Box 32, 60 Murray Street, Toronto, Ontario M5T 3L9, Canada. Tel.: +1 416 586 8443; fax: +1 416 619 5521.
E-mail addresses: jane.bayani@utoronto.ca (J. Bayani), paula.marrano@sickkids.ca (P. Marrano), cassy.graham@utoronto.ca (C. Graham), yzheng@fhcrc.org (Y. Zheng), linl@fhcrc.org (L. Li), dhocc@libero.it (D. Katsaros), heini.lassus@helsinki.fi (H. Lassus), butzow@mappi.helsinki.fi (R. Butzow), squirej@queensu.ca (J.A. Squire), ediamandis@mtsinai.on.ca (E.P. Diamandis).
1574-7891/$ – see front matter
doi:10.1016/j.molonc.2010.08.002

correlated to tumor grade ($p < 0.001$). We conclude that numerical and structural aberrations of chromosome 19q, affect genes including the *KLK* gene members, may contribute to ovarian carcinoma progression and aggressiveness.

1. Introduction

Ovarian carcinoma (OCa) is a heterogenous disease, reflected by distinct histopathological subtypes, and clinically characterized according to stage and grade (reviewed by Bast et al. (2009)). A growing body of evidence suggests distinct molecular pathways may mediate the development and characterization of low-grade and high-grade serous tumors; among them, the relative differences in genomic instability and mutations of *PTEN* and *KRAS* in low-grade tumors; and mutations and aberrant expression of *TP53* among high-grade tumors (Bast et al., 2009; Ricciardelli and Oehler, 2009; Shih Ie and Kurman, 2004). Genomically, OCas exhibit complex numerical and structural chromosomal alterations (Bayani et al., 2002; Pejovic et al., 1992a,b; Shridhar et al., 2001; Taetle et al., 1999a,b; Thompson et al., 1994a,b), and are prone to chromosomal instability (CIN) (Bayani et al., 2008a; Gorringe et al., 2005; Kuo et al., 2009). CIN is mediated by various mechanisms (reviewed by Bayani et al. (2007)), resulting in chromosomal abnormalities which can be broadly classified as numerical chromosomal instability (N-CIN) or structural chromosomal instability (S-CIN) (Bayani et al., 2007). The consequences of gross numerical and structural chromosomal rearrangements include changes in gene sequence, gene/locus-specific dosage (Frohling and Dohner, 2008), as well as epigenetics (Sadikovic et al., 2008); which all contribute to the pathogenesis of this disease. Chromosome 19q is a site of frequent rearrangements (http://cgap.nci.nih.gov/Chromosomes/Mitelman), and copy-number imbalances (http://www.progenetix.net/) in many neoplasms, including OCa. Classical cytogenetic analyses (Taetle et al., 1999a,b), and more recent molecular cytogenetic findings (Micci et al., 2009; Tsao et al., 2001) suggest that the non-random numerical and structural alterations of chromosome 19 play an important role in ovarian carcinogenesis.

In a small pilot study, we recently investigated the role of copy-number of the kallikrein (*KLK*) genes in a series of cancer cell lines and primary ovarian cancers shown to possess KLK6 overexpression (Bayani et al., 2008b). In all primary ovarian cancer specimens, a net gain of the entire *KLK* locus (19q13.3/4) was identified, either by the whole gains of chromosome 19 or through unbalanced translocations involving breakpoints centromeric to 19q13.3/4, suggesting a role of copy-number in the observed elevated KLK6 protein levels. The *KLKs* are a family of 15 genes and 1 pseudogene located in tandem on chromosome 19q13.3/4 (Yousef et al., 2000), which encode trypsin-like serine proteases. They cleave a variety of substrates including MMPs, IGFBPs, fibronectins and collagens (Borgono and Diamandis, 2004) and seem to be involved in many of the pathways deemed the hallmarks of cancer (Hanahan and Weinberg, 2000). Due to the widespread and successful use of *PSA* (also known as *KLK3*) as a biomarker for the detection and monitoring of prostate cancer (Lilja et al., 2008), the potential for other *KLKs* as biomarkers in ovarian (Kim et al., 2001; Kyriakopoulou et al., 2003; Shan et al., 2006; Yousef et al., 2003b) and other cancers has been actively pursued (Inoue et al., 2010; Li et al., 2009b; Mavridis and Scorilas, 2010; Nathalie et al., 2009; Pettus et al., 2009; Shinoda et al., 2007; Planque et al., 2005). The observed differential expression of the KLK genes has also fueled numerous studies investigating the mechanism regulating their expression, including hormone stimulation (Lai et al., 2009; Lawrence et al., 2010; Shan et al., 2007; Shaw and Diamandis, 2008), differential methylation (Pampalakis et al., 2008, 2009; Pampalakis and Sotiropoulou, 2006; Sidiropoulos et al., 2005) and microRNAs (miRNAs) (Chow et al., 2008; White et al., 2010), with few studies investigating copy-number (Bayani et al., 2008b; Ni et al., 2004; Shinoda et al., 2007). These variable findings suggest that the *KLKs* may be regulated by multiple mechanisms.

Among the *KLK* gene family, *KLK6* has been demonstrated to be a promising biomarker for OCa (Borgono et al., 2006; Hoffman et al., 2002; Obiezu et al., 2001; Shan et al., 2007; White et al., 2009; Yousef et al., 2003a). Recent studies by our group (Shan et al., 2007) have shown up to 57-fold higher KLK6 protein expression in cancer specimens compared to normal tissues; with univariate and multivariate survival analyses showing that patients deemed KLK6-positive, are at an increased risk of relapse, in comparison to KLK6-negative patients. A significant association with progression-free survival (PFS) and overall survival (OS) was also demonstrated.

While the assessment of KLK6 as a novel biomarker in OCa continues, the mechanisms leading to the observed protein overexpression of KLK6 are still unclear (Borgono et al., 2006). Our previous findings (Bayani et al., 2008b) suggest that copy-number may contribute to such overexpression, and the numerical and structural complexity of OCa karyotypes influences copy-number change. Thus, in this investigation, we have expanded the study to specifically investigate the overall frequency of *KLK* locus-specific copy-number changes, and to correlate these findings to KLK6 expression in a series of untreated serous ovarian carcinomas. Furthermore our experimental design enabled us to examine the frequency of structural and numerical CIN on 19q, and its implications for understanding ovarian cancer pathogenesis.

2. Materials and methods

2.1. Patient specimens

Seventeen formalin-fixed paraffin embedded (FFPE) sections were obtained from the Department of Gynecology and Obstetrics, University of Turin, Italy; and an ovarian tissue microarray (TMA), consisting of 102 patients was obtained from the Department of Pathology at the Helsinki University

Central Hospital (C2 block); and were collected with consent according to the guidelines of research ethics boards from all institutions. All specimens were classified histologically as serous tumors derived from patients naïve to chemotherapy. The tissues were fixed according to standard procedures and the TMA was constructed as previously described (Lassus and Butzow, 2007). FFPE normal ovarian tissues were obtained from US Biomax (Rockville, MD, catalog number HuFPT076).

2.2. *Fluorescence* in situ *hybridization (FISH)*

For copy-number and instability studies, the following bacterial artificial chromosome (BAC) clones mapping to three regions of chromosome 19q were obtained from The Centre for Applied Genomics (Toronto, Canada): 19q12 (RP11-888D2, RP11-197B9, RP11-716O8 and RP11-1107F24), 19q13.2 (RP11-67A5, RP11-264N23, RP11-825A10), and 19q13.3/4 (RP11-288H1, RP11-10I11, RP11-615L12). The 19q13.3/4 overlapping BACs span the entire *KLK* locus to include *KLK1* at the most centromeric end to span all *KLK* gene family members; and including *CD33* at the telomeric end. To confirm that *KLK6* was represented in the overlapping BAC clones RP11-10I11 and RP11-615L12, *KLK6*-specific PCR was performed using the following primer set designed using Primer 3 (http://frodo.wi.mit.edu/_): *KLK6* forward GGGGTCCT TATCCATCCACT and *KLK6* reverse CAGTCGCATCTGCTGTTCAT, to yield a 90 bp band. Using the Illustra Hot Start Master Mix (GE Healthcare Life Sciences), 100 ng of BAC DNA were tested, in addition to proper positive and negative controls. DNA extracted from the BAC clones was labeled with Spectrum Green (Vysis/Abbott Laboratories, Des Plaines, IL), Spectrum Orange (Vysis/Abbott), or blue-fluorescing DEAC (Applied Biosystems) by nick translation using the Vysis Nick Translation Kit (Vysis/Abbott), according to the manufacturer's instructions. FISH to normal human lymphocytes (Bayani and Squire, 2004c) confirmed the genomic location of all BACs. All 19q12 overlapping clones (RP11-888D2, RP11-197B9, RP11-716O8 and RP11-1107F24) were labeled with Spectrum Green. Similarly, all 19q13.2 clones were labeled with DEAC; and all 19q13.3/4 clones which span the KLK locus, were labeled with Spectrum Orange. Approximately 300 ng of each labeled DNA were precipitated in excess Human Cot-1 DNA (Invitrogen, Canada), sonicated salmon sperm DNA (Roche, Canada) and resuspended in a 50% formamide/10% dextran sulfate/2× SSC hybridization buffer (DAKO, Mississauga, ON, Canada) to a final volume of 35 μl (Bayani and Squire, 2004b). All tissues were baked overnight at 56 °C. The following day, the slides were de-waxed in xylene and dehydrated in 100% ethanol and pre-treated prior to co-denaturation and hybridization with the probe cocktail. The following day, the slides were processed in a wash of 0.3% NP-40/0.4× SSC for 2 min at 72 °C and a wash of 0.1%NP-40/2× SSC for 5 min at RT. The slides were rinsed in 1× PBS, mounted in a DAPI/Antifade medium (Vectashield/Vector Laboratories Canada), and visualized at 60× with a Zeiss Axioskop fluorescence microscope (Carl Zeiss Canada).

2.3. *Measurement of chromosomal instability*

For each case/core at least 50–100 tumor nuclei were scored for the presence of each signal. A normal diploid cut-off was established using normal tissues, resulting in at least 65% of cells with 2 signals for each probe and 35% containing 1 signal (due to tissue sectioning). Greater than 40% of cells with 1 signal indicated the presence of a population with a net loss. The presence of additional populations were noted when the frequency of positive cells exceeded 10% per population (or cumulatively exceeding 10% if several populations were detected). Using a modification of chromosomal instability index previously published (Bayani et al., 2008a), the designation of low, medium or high chromosomal instability (CIN) was assigned when 1 or 2 populations of cells were identified (low), 2–3 populations of cells were identified (medium), or greater than 3 populations of cells or amplification (high) were detected. *Numerical changes of 19q*: Whole copy-number changes of 19q were identified when the percentage of signals per loci along 19q were similar. *Structural changes of 19q*: Evidence of structural rearrangement on 19q was identified when the percentage of signals per cell, for each of the loci tested, were discordant by greater than 10%.

2.4. *Immunohistochemistry (IHC)*

Immunohistochemical analyses using a mouse monoclonal (1:150), and rabbit poly-clonal (1:1000) antibody with high specificity for KLK6 (Diamandis et al., 2000) was performed as described previously by Petraki et al. (2001, 2006). IHC for the detection of both wildtype and mutant p53 protein was performed using a monoclonal antibody (DO-7, 1:25 Dako Canada, Mississauga, ON.) according to the manufacturer's instructions. Since wildtype p53 possesses a short half-life, its presence in minute amounts is below the level of detection for IHC (Vojtesek et al., 1992). Thus, the accumulation of p53 protein detected by IHC reflects aberrant p53 forms. Briefly, tissue sections were de-waxed in xylene, dehydrated in 100% ethanol and rinsed in water. Antigen retrieval was performed by pressure cooking in 0.01M citrate buffer for 30 min. The slides were rinsed in water and treated for 30 min in 0.3% hydrogen peroxide/methanol. The slides were rinsed in water and processed using the ImPRESS Detection Kit (Vector Laboratories). Following a 20 min incubation with normal horse serum, both KLK6 antibodies diluted in DAKO diluents, or p53 antibody, were applied to the slides for 1 h at room temperature. The slides were incubated with the appropriate secondary anti-rabbit and anti-mouse antibodies for 30 min, washed, treated with the polymer reagent, and then detected with standard DAB solution for 5 min. The slides were stained with hematoxylin, and finally mounted in Permount (Fisher Scientific Canada). No antibody controls were also performed. The slides were scanned using the Aperio ScanScope (Aperio Technologies Inc, San Diego, CA) for visualization and image acquisition. Standard hematoxylin- and eosin-stained slides were also scanned for visualization. KLK6 positivity was assessed according to overall staining intensity (1 = low, 2 = medium, 3 = high) compared to surface epithelial cells from a normal ovarian tissue control and in "no antibody" controls. Similarly, p53 expression was assessed against the surface epithelial cells from a normal ovarian tissue control and "no antibody" controls, and scored according to staining intensity and cell distribution (1 = normal; negative/low staining with <20% cells positive; 2 = moderate; moderate staining with >50% cells positive; 3 = strong; strong positivity with >50% cells with strong staining).

2.5. Statistical methods

The associations between outcome measurements: 19q rearrangement, *KLK* copy-number, chromosomal instability and p53 immunohistochemical intensity and clinical parameters were examined by ANOVA, two-sample *t*-test and Chi-square test, as deemed appropriate. Cox survival analysis was applied to disease-free survival (DFS) and overall survival outcomes both univariately and multivariately. In the multivariate survival analyses, only the clinical parameters that were shown to be independent were included for adjustment. All statistical analyses were performed using SAS software (version 9.2; SAS Institute). Two-sided *P*-values for statistical significance were set at <0.05.

3. Results

3.1. Patient cohort

The entire patient cohort consisted of 119 serous ovarian carcinoma tissues from patients naïve to chemotherapy. The final number of patients available for detailed analysis was 81, due either to poor tissue quality or lack of sufficient clinical data. The median age at diagnosis was 56.2 years. Tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria and graded according to Day et al. (Day et al., 1975). Thirteen tumors were classified as stage I, 6 as stage II, 50 as stage III and 16 as stage IV, with one unknown stage. Four cases were grade 0 and included samples with evidence of malignancy, 22 were grade I, 15 were grade II and 40 cases were grade III. Response was classified as complete, partial, progressive or stable at follow-up within 6 months. The median follow-up time was 72 months.

3.2. The KLK locus (19q13.3/4) is subject to frequent copy-number alterations and chromosomal instability

All 19q bacterial artificial chromosome (BAC) clones used in this study were individually FISH-mapped to normal human metaphase chromosomes (data not shown) to ensure the proper mapping location and absence of cross-hybridization to other chromosomes. The multi-colour FISH strategy is illustrated in Figure. 1A, with 19q12 probes, near the centromere labeled in green; 19q13.2 probes, approximately 11 MB telomeric to the 19q12 BACs labeled in blue; and the *KLK* locus at 19q13.3/4, a further 10 MB telomeric to the 19q13.2 BACs, labeled in red. *KLK6*-specific PCR confirmed the presence of *KLK6* in BAC clones RP11-10I11 and RP11-615L12 (Figure. 1A). Of the 119 specimens available, 81 were successfully analysed by FISH for copy-number changes of the KLK locus. Across all cases, 14 showed 2 copies of the *KLK* locus (17.3%); 24 showed a net loss of the locus (29.6%), 41 (50.6%) showed a net gain of the *KLK* locus, and 2 (2.5%) showed high-level amplification (Figure. 1B, Table 1). These findings indicated that 82.7% of cases exhibited copy-number alterations (ie. gain, loss, or amplification) at the 19q13.3/4 locus, containing the *KLK* genes. Moreover, because three genomic regions on 19q were enumerated (19q12, 19q13.2 and 19q13.3/4), we could assess whether such copy-number gains or losses were due to whole gains of 19q or through structural rearrangements along 19q. The presence of structural alterations on 19q was identified when the percentage of signals per cell, per 19q locus scored, was discordant. In contrast, when there were similar frequencies of signals per cell across all genomic loci, the 19q arm was considered intact (Figure. 1C). Of the 14 cases which showed two copies of the *KLK* locus per cell, 4 (28.6%) were involved in a structural rearrangement on 19q and the remaining 10 cases (71.4%) showed no indication of structural rearrangement of 19q. Of the 24 cases showing a net loss of the *KLK* locus, 18 (75%) were a consequence of structural rearrangements of 19q, while the remaining 6 (25%) were due to the entire loss of the 19q arm. Twenty-five of the cases showing net gains of the *KLK* locus (61.0%) were a result of structural aberrations of 19q, while 16 of the cases (39%) showed gains of the *KLK* locus through whole 19q arm copy-number gains. Finally, the two cases which possessed amplification of the *KLK* locus, resulted from structural aberrations of 19q (100%). Examples of these different classes of genomic changes are illustrated in Figure. 1C.

It was evident from these results that the copy-number changes were often heterogeneous. In many cases a predominant clone was present and accompanied with lesser populations, all contributing to the net gain or loss of the locus. The extent of numerical CIN of the *KLK* locus was assessed by enumerating the number of populations (greater than 10% in frequency). In all cases in which only 2 copies of the *KLK* locus were identified, low-level CIN was observed; that is, there was presence of one or two populations of cells. Similarly, cases showing the net loss of the locus were also characterized primarily as low-level CIN. However, unlike 2-copied and net-deleted cases, the observed net gains of the *KLK* locus were characterized predominantly by moderate-CIN (up to 3 populations of cells) and high-level CIN (>3 populations), particularly among those cases where 19q rearrangements were detected. In those net *KLK* gained cases resulting from whole 19q arm gains, the level of CIN ranged from low to moderate (Table 1).

The correlation of *KLK* locus copy-number to clinical parameters is summarized in Table 1. Across age, stage, grade, response to treatment, KLK6-specific expression and extent of chromosomal instability, statistical significance was shown only for chromosomal instability. Low instability was associated with cases showing only 2 copies of the *KLK* locus, with copy-number gains and losses associated with increasing instability ($p < 0.001$).

3.3. Structural alterations of chromosome 19q are associated with age and grade

When the tumors were classified as either possessing 19q rearrangements or without rearrangements, 61.7% (50/81) possessed 19q rearrangements and the remaining 38.3% (31/81) had no apparent gross structural rearrangements (Figure. 1B). The ranges of copy-number gains and losses for the genomic loci analysed were primarily low. Losses were typically characterized by the loss of one copy from the primary clonal populations; and gains were characterized by one to three extra copies compared to the primary clonal population. As mentioned previously, the *KLK* locus was amplified in 2 of the rearranged

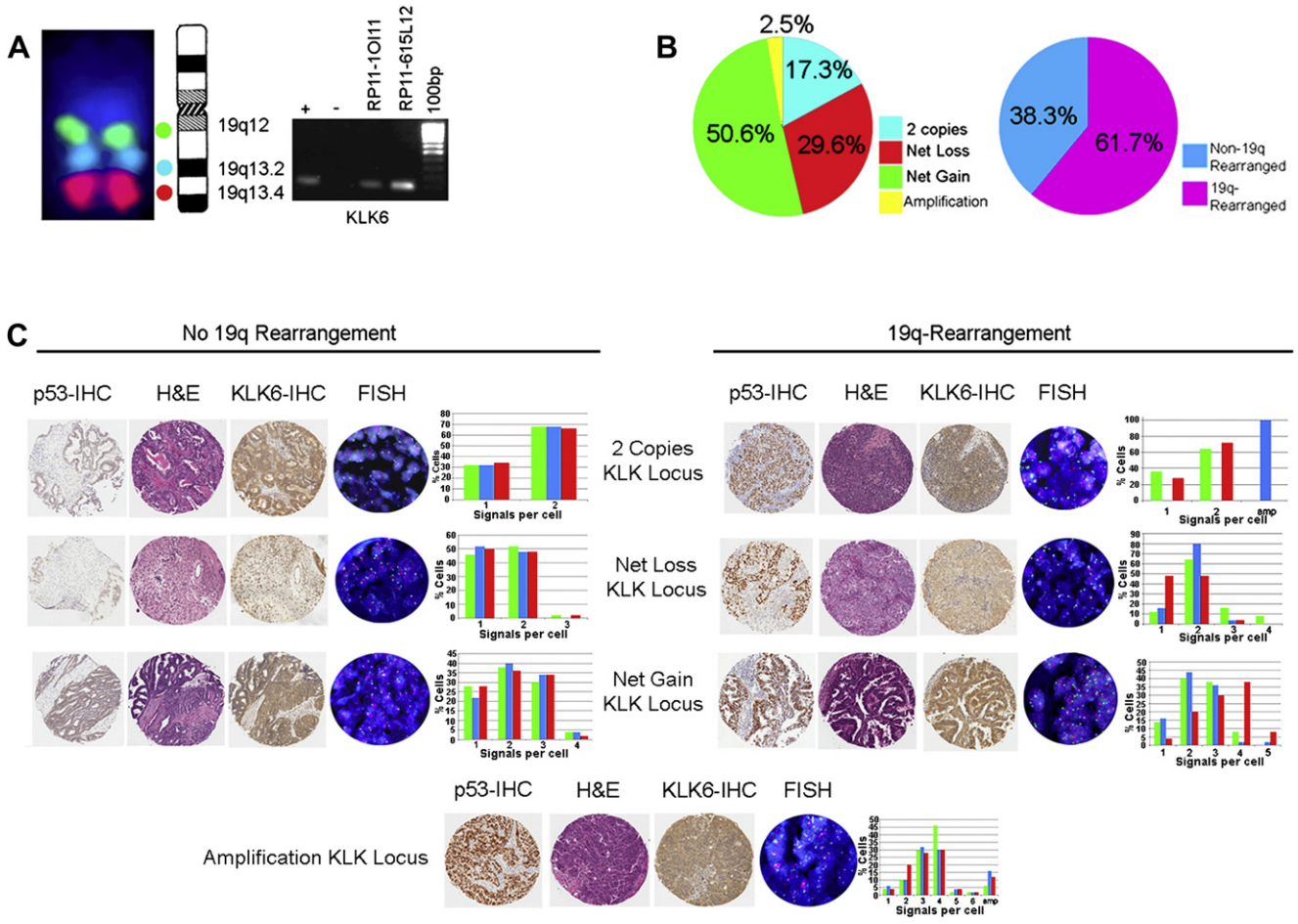

Figure 1 – KLK Copy-Number Analysis of Serous Ovarian Carcinomas by FISH and Immunohistochemistry. A. Multi-colour FISH strategy. Shown is a normal chromosome 19 with the mapping location of the BAC clones used and PCR confirmation for the presence of *KLK6* in BAC clones RP11-1OI11 and RP11-615L12. Also shown are "+" (positive) and "−" (negative) control lanes. The 19q12 locus is identified by green signals, 19q13.2 by blue signals and 19q13.3/4 (*KLK* locus) by red signals. B. Left panel: Distribution of copy-number changes of the *KLK* locus in 81 cases shows that the KLK locus (19q13.3) is subject to frequent copy-number changes which are dominated by gains/amplifications. Right panel: Classification of the presence or absence of 19q rearrangement reveals that 19q is frequently subject to structural aberrations. C. Representative images of IHC for p53 and KLK6 including hematoxylin/eosin (H&E), together with corresponding FISH images depicting each of the 4 classes of copy-number changes of the *KLK* locus and the two rearrangement classes. The 19q12 locus is identified by green signals, 19q13.2 by blue signals and 19q13.3/4 by red signals. The corresponding histograms show the range in number of signals per cell for each genomic locus. Rearranged cases were identified when the frequency of signals for each of the genomic loci were discordant. Cases were identified as not possessing rearrangements on 19q when the frequency of signals across all genomic loci tested per cell was similar.

cases; and 19q13.2 amplification was seen in 9/50 (18%) of rearranged cases (or 9/81 (11.1%) of total cases). In 6 of the 9 cases, 19q13.2 was amplified in 100% of cells scored, whereas the remaining 3 cases showed amplification of 19q13.2 as an additional population to polysomy for the locus, indicating genomic heterogeneity. Amplification of 19q12 was also detected in 2/50 (4%) of rearranged cases, but as additional populations to polysomy for the locus (Figure. 1C).

When 19q rearrangement status was correlated to the same clinical parameters (Table 2), grade was found to be significant ($p \leq 0.001$), with 58% of the low-grade tumors showing no 19q rearrangements. This was in contrast to the high-grade tumors in which 84% of cases possessed 19q rearrangements. Age was also significant ($p = 0.003$), revealing that 19q rearrangements occurred more frequently in older patients.

3.4. *Serous ovarian carcinomas show a range of KLK6 and p53 expression*

Since KLK6 protein overexpression has been observed in ovarian carcinomas, immunohistochemical analysis was performed, revealing a range of KLK6 overexpression. Normal ovarian surface epithelium expressed KLK6 at a barely detectable level by IHC, consistent with previous findings (Ni et al., 2004; Petraki et al., 2001). Positive controls were derived from formalin-fixed paraffin embedded (FFPE) sections with accompanying KLK6 quantification by ELISA from tissue extracts, as previously described (Shan et al., 2007). All 81 patient specimens showed positive cytoplasmic staining for KLK6, ranging from low-level to very high expression (Figure. 1C). Stromal components did not express KLK6. The comparison of the overall

Table 1 – Clinical characteristics of patients stratified by *KLK* copy-number.

		KLK Copy-Number			
		2 Copies ($n = 14$)	Net Loss ($n = 24$)	Net Gain ($n = 43$)[a]	p-value*
Age (years)	Mean (SD)	57.1 (16.1)	51.5 (10.9)	58.6 (13.3)	0.113
Stage	I/II	2 (14%)	7 (29%)	10 (24%)	0.582
	III/IV	12 (86%)	17 (71%)	32 (76%)	
Grade	0/I	6 (43%)	8 (33%)	12 (28%)	0.575
	II/III	8 (57%)	16 (67%)	31 (72%)	
Response	Complete	11 (79%)	16 (67%)	20 (48%)	0.271
	Partial/Stable	2 (14%)	6 (25%)	15 (36%)	
	Progression	1 (7%)	2 (8%)	7 (17%)	
Instability	Low	14 (100%)	18 (75%)	14 (33%)	<0.001
	Mod/High	0 (0%)	6 (25%)	29 (67%)	
P53-IHC	Normal	5 (36%)	7 (29%)	10 (24%)	0.672
	Mod/Strong	9 (64%)	17 (71%)	32 (76%)	
KLK6-IHC	Low/Mod	4 (29%)	11 (46%)	12 (28%)	0.301
	Strong	10 (71%)	13 (54%)	31 (72%)	

*p-value from t-test and Chi-square test.
a Net Gain includes amplified cases.

intensity of KLK6 staining to *KLK* locus copy-number showed no clear relationship (Table 1, Figure. 1C). Strong expression of KLK6 was found in the majority of cases with two copies of the locus (10/14); as was also the case for specimens with a net gain of the locus (31/43). Cases with a net loss of the locus showed roughly equal numbers of specimens with low/moderate and strong staining (11/24 and 13/14, respectively). In the two cases which were shown to be amplified, strong cytoplasmic staining was shown in both cases.

Immunohistochemical analysis of p53 was successful in 80/81 specimens. Normal ovarian tissue showed negative to very weak reactivity for p53 with less than 20% of cells showing weak positivity, corresponding to the normally short half-life of wildtype p53 (Vojtesek et al., 1992). Twenty-two cases (27.5%) were identified as having reactivity consistent with normal p53 protein expression; whereas abnormal p53 was identified in 22 cases (27.5%) showing moderate staining, and in 36 cases (45%) showing strong reactivity, indicating abnormal p53 accumulation. When p53-IHC was correlated to age, stage, grade, response, *KLK* copy-number, chromosomal instability and KLK6 IHC results, only grade was found to be statistically significant ($p = 0.040$) with higher grade tumors associated with stronger p53 staining.

Table 2 – Clinical characteristics of patients stratified by 19q rearrangement.

		19q Rearranged		
		No ($n = 31$)	Yes ($n = 50$)	p-value*
Age (years)	Mean (SD)	50.8 (13.3)	59.6 (12.3)	0.003
Stage	I/II	10 (33%)	9 (18%)	0.119
	III/IV	20 (67%)	41 (82%)	
Grade	0/I	18 (58%)	8 (16%)	<0.001
	II/III	13 (42%)	42 (84%)	
Response	Complete	20 (65%)	27 (55%)	0.618
	Partial/Stable	7 (23%)	16 (33%)	
	Progression	4 (13%)	6 (12%)	

*p-value from t-test and Chi-square test.

3.5. *Univariate and multivariate analyses*

When hazard ratios (HR) estimated from the Cox regression model for disease-free survival (DFS) were calculated at both univariate and multivariate levels (Table 3), response to chemotherapy was highly significant (complete response HR = 1; partial response/stable disease HR = 3.19; progression, HR = 6.96, $p \leq 0.001$) for both univariate and multivariate analyses. Similar data were seen for stage and grade (Table 3). Age was significant only in univariate analyses ($p = 0.026$). Although hazard ratios based on *KLK* copy-number (2 copies, HR = 1; Net loss, HR = 0.78; Net gain, HR = 1.2, $p = 0.441$), or KLK6 IHC (low, HR = 1; Mod/High, HR 1.9, $p = 0.557$) were not statistically significant on univariate and multivariate analyses, Kaplan–Meier curves for DFS displayed a trend for those patients with a net loss of the locus to have a relatively better DFS over patients with 2 copies or gains of the locus (Figure. 2A). Hazard ratios for DFS revealed that 19q rearrangement status was marginally significant (no rearrangement, HR = 1; 19q rearrangement, HR = 1.68; $p = 0.078$) in univariate analysis, but not significant in multivariate analysis. Furthermore, p53-IHC was also not significant on univariate ($p = 0.458$) or multivariate ($p = 0.554$) for DFS.

For overall survival (OS) (Table 4), chemotherapy response was highly significant in both univariate and multivariate analyses ($p < 0.001$), as was grade ($p < 0.001$ and $p = 0.002$, respectively) and age ($p < 0.001$ and $p = 0.021$, respectively). Stage was only significant in univariate analysis ($p = 0.009$). Weak associations based on 19q rearrangement and p53

Table 3 – Hazard ratio (HR) estimated from the Cox regression model for disease-free survival (DFS).

		Univariate		Multivariate*	
		HR	P	HR	P
Age (years)		1.02	0.026	1	0.792
Stage	I/II	1	0.002	1	0.011
	III/IV	3.65		3.13	
Grade	0/I	1	0.001	1	0.001
	II/III	3.58		4.19	
Response	Complete	1	<0.001	1	<0.001
	Partial/Stable	3.19		3.29	
	Progression	6.96		6.64	
KLK6-IHC	Low	1	0.557	1	0.879
	Mod/High	1.19		0.95	
19q rearranged	No	1	0.078	1	0.756
	Yes	1.68		0.9	
KLK Copy-Number	2 Copies	1	0.441	1	0.812
	Net Loss	0.78		0.78	
	Net Gain	1.2		0.94	
Instability	Low	1	0.302	1	0.677
	Mod/High	1.33		1.13	
p53-IHC	Normal	1	0.458	1	0.554
	Mod/Strong	1.27		1.22	

* Multivariate Cox model for 19q-rearranged, KLK copy-number, chromosomal instability, and p53-IHC includes: stage, grade and chemotherapy response. Mod = moderate.

protein status were seen only in univariate analyses ($p = 0.068$ and $p = 0.077$, respectively). Neither *KLK* copy-number nor KLK6-specific IHC was significant for OS on univariate or multivariate analyses However, as demonstrated for DFS, Kaplan–Meier curves (Figure. 2B) for 19q rearrangement, *KLK* copy-number and p53 expression, show a trend for better overall survival in patients who had no rearrangements in 19q; who had a net loss of the KLK locus or with normal p53 expression.

4. Discussion

Ovarian carcinoma is an aggressive disease with a poor outcome. The search for biomarkers, not only for diagnosis, but also for better prediction of outcome and response to therapy is of paramount importance. Although CA 125 is used for monitoring disease recurrence following surgery and chemotherapy, it lacks specificity as an early detection diagnostic biomarker (Bast et al., 2009). Potential new biomarkers for OCa include members of the kallikrein gene family (*KLK*) which maps to 19q13.3/4 (Borgono and Diamandis, 2004; Yousef et al., 2000). Among them, overexpression of KLK6 protein, as detected by tissue extract enzyme-linked immunosorbent assay (ELISA) has been demonstrated to be an important prognostic marker (Shan et al., 2007). The mechanisms leading to the observed KLK6 overexpression are still unclear (Borgono and Diamandis, 2004), though hormone stimulation (Lai et al., 2009; Lawrence et al., 2010; Shan et al., 2007; Shaw and Diamandis, 2008), differential methylation (Pampalakis et al., 2008, 2009; Pampalakis and Sotiropoulou, 2006; Sidiropoulos et al., 2005) and regulation by microRNAs (miRNAs) (Chow et al., 2008; White et al., 2010) have been investigated. Based on our previous preliminary findings (Bayani et al., 2008b), we have further investigated the role of *KLK* copy-number in 81 untreated serous ovarian cancers using a three-colour FISH approach targeting loci on 19q, including the *KLK* locus, which harbours *KLK6*, and performing KLK6-specific immunohistochemistry (IHC) to investigate the relationship between *KLK* copy-number and KLK6 protein expression. In this way, we were also able to determine whether such copy-number imbalances were the result of whole chromosomal gains of 19q, or disruption of 19q; as alluded to in early and more recent studies implicating abnormalities of chromosome 19 (particularly 19q13), in ovarian cancer pathogenesis (Micci et al., 2009; Taetle et al., 1999b; Thompson et al., 1996; Tsao et al., 2001).

Of the 81 tumors, we found that the *KLK* locus, using BAC clones confirmed to contain *KLK6*, was subject to changes in copy-number (gain, loss or amplification) in 82.7% of cases, with gains/amplifications (53.1%) being more common over losses (29.6%). Only 2.5% of cases showed high-level amplification and 17.3% harboured only 2 copies. Consistent with our previous cytogenetic data (Bayani et al., 2008b), we found that the gains/amplifications of the *KLK* locus resulted primarily from structural rearrangements of 19q. The same observation was made for tumors showing net loss of the *KLK* locus, and this was in contrast to tumors where only two copies of the *KLK* locus were identified. Many carcinomas (Al-Romaih et al., 2003; Bayani et al., 2003; Maire et al., 2009; Vukovic et al., 2007), including ovarian, show karyotypic heterogeneity both numerically and structurally (Bayani et al., 2002, 2008a; Gorringe et al., 2005); and such on-going chromosomal instability (CIN) and genomic variability is believed to drive tumor progression (Li et al., 2009a). Indeed, our analyses revealed CIN and copy-number heterogeneity of the *KLK* locus ($p < 0.001$), illustrated by the observed number of subpopulations of cells with greater or fewer *KLK* locus signals, in addition to the primary clonal population (Figure. 1). Hazard ratio and Cox regression analyses for DFS based on *KLK* copy-number status showed no significant correlation in univariate and multivariate analyses ($p = 0.441$ and $p = 0.812$, respectively). However, Kaplan–Meier analysis suggested a trend for increasing DFS among those cases with net loss of the *KLK* locus; whereas, patients with 2 copies, or those who were shown to possess gains/amplifications had similar DFS.

KLK6-specific IHC analyses revealed overexpression in all cases ranging from weak to intense immunoreactivity. Although there was no correlation between copy-number and protein expression ($p = 0.301$), there was a trend suggesting that those tumors with a gain of the locus were more likely to overexpress KLK6. When only two copies were identified, the majority of cases expressed KLK6 levels with equal frequencies of moderate to low expression. This suggests that copy-number could contribute somewhat to protein expression, but is not the primary regulatory mechanism, as exemplified by the cases with two copies, or loss of one copy, but still exhibiting strong KLK6 expression; or cases with gain/amplification of the locus and relatively moderate expression. No

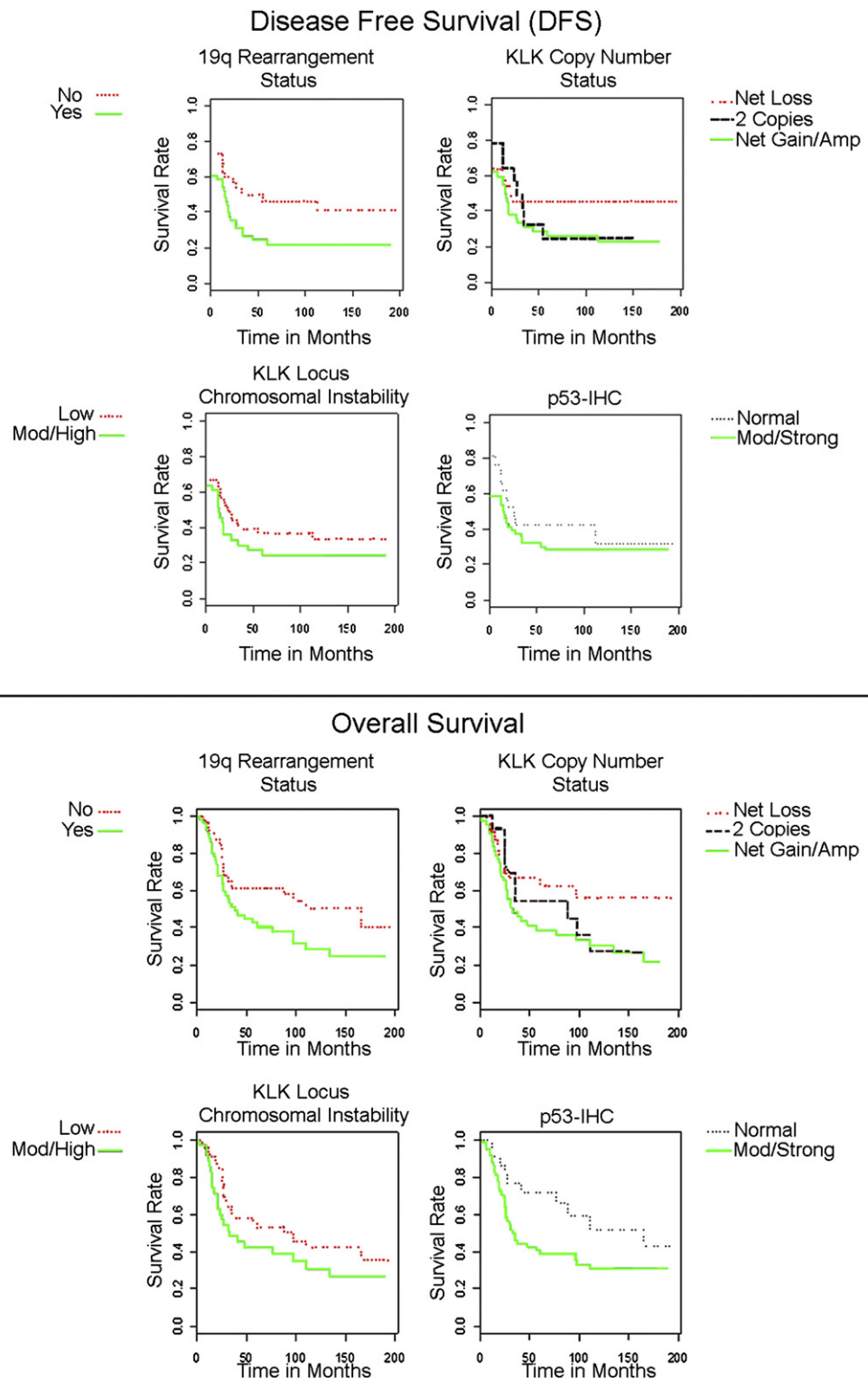

Figure 2 – Kaplan–Meier Curves for Disease-Free Survival (DFS) and Overall Survival (OS). Shown are the Kaplan–Meier curves (unadjusted) based on 19q rearrangement status, *KLK* copy-number status, *KLK* locus chromosomal instability and p53-IHC. For both DFS and OS, patients with no 19q rearrangement had a better outcome than those with disruptions of 19q. Similarly, a trend could be seen for better DFS and OS in patients with a net loss of the *KLK* locus.

relationship with respect to DFS ($p = 0.557$), was observed based on KLK6 IHC expression, in keeping with the results of another study (Kountourakis et al., 2008), but in contrast to Shan et al. (2007), suggesting ELISA-based measurements of protein levels may be more informative. Thus, the results of the KLK6 immunohistochemical and copy-number analyses point to additional mechanisms contributing to KLK6 protein overexpression.

Interestingly, when tumors were stratified as 19q rearranged or non-19q rearranged, 61.7% of cases showed

Table 4 – Hazard ratio (HR) estimated from the Cox regression model for overall survival (OS).

		Univariate		Multivariate*	
		HR	P	HR	P
Age		1.04	<0.001	1.03	0.021
Stage	I/II	1	0.009	1	0.237
	III/IV	2.94		1.75	
Grade	0/I	1	<0.001	1	0.002
	II/III	5.22		4.03	
Response	Complete	1	<0.001	1	<0.001
	Partial/Stable	2.89		2.83	
	Progression	13.82		11.92	
KLK6-IHC	Low	1	0.498	1	0.574
	Mod/High	1.23		1.2	
19q-rearranged	No	1	0.068	1	0.909
	Yes	1.75		1.04	
KLK Copy-Number	2 Copies	1	0.109	1	0.718
	Net Loss	0.59		0.7	
	Net Gain	1.26		0.93	
Instability	Low	1	0.163	1	0.512
	Mod/High	1.49		1.22	
p53-IHC	Normal	1	0.077	1	0.545
	Mod/Strong	1.88		1.25	

* Multivariate Cox model for 19q-rearranged, KLK copy-number, chromosomal instability, and p53-IHC includes: age, grade and chemotherapy response. Mod = moderate.

structural abnormalities at 19q; which were strongly associated with tumor grade ($p < 0.001$). Fewer low-grade tumors possessed structural rearrangements of 19q (16%) as compared to their high-grade counterparts (84%). Moreover, correlation to DFS and OS showed that patients with 19q rearrangements had a shorter DFS and worse OS, which were of boarder line significance ($p = 0.078$ and $p = 0.068$ respectively) in univariate analyses. These combined findings provide some credence to the two pathway model for ovarian tumorigenesis, where type I ovarian tumors are classified as low-grade, slow-growing, with low-level and increasing CIN; and type II tumors, are classified as higher grade, more rapidly growing, with high CIN, and with a much lower 5-year survival (Ricciardelli and Oehler, 2009; Shih Ie and Kurman, 2004). Our study demonstrated that tumors classified as low-grade (grade 0/I) had fewer structural and range of numerical abnormalities of chromosome 19 than high-grade tumors (grade II/III) (Table 2); supporting the notion that changes on chromosome 19q, or the molecular pathways influencing the observed numerical and structural changes on chromosome 19q, may play a role in OCa pathogenesis. Indeed, Type I tumors more frequently possess mutations in *KRAS* and *PTEN*, and are subject to greater microsatellite instability (MSI), whereas Type II tumors frequently possess mutations in *p53* and *BRCA1/2*, and are subject to CIN. Our p53-IHC findings are consistent with this model, with high-grade tumors more frequently showing overexpression for p53 over low-grade tumors ($p = 0.040$). The contribution of *TP53* to chromosomal instability has been well-documented (Fukasawa, 2005; Tomasini et al., 2008). Thus, the concurrent findings of genomic instability and p53 overexpression, in high-grade cases is consistent with previous reports (Blegen et al., 2000; Ceccaroni et al., 2004; McManus et al., 1996). Although some studies have shown correlation of p53 expression and overall poor outcome and survival (Lassus and Butzow, 2007), our analyses showed only a weak association with poor overall survival ($p = 0.077$), likely due to our smaller sample size.

To our knowledge, this is the first report suggesting that both structural and numerical abnormalities of chromosome 19 distinguish low-grade and high-grade cancers. Further analyses on a larger cohort of tumors using the specific markers distinguishing Type I and Type II tumors (Ricciardelli and Oehler, 2009; Shih Ie and Kurman, 2004) is warranted to determine whether the preliminary observations seen here are associated. Indeed, early classical cytogenetic studies have demonstrated OCa karyotypes are numerically and structurally complex, with a propensity for the non-random gain and loss of specific chromosomes, and the apparent preferential sites of chromosomal breakage (Taetle et al., 1999a,b), including 1p1*, 1q1*, 1p2*, 1q2*, 1p3*, 1q3, 3p1*, 1q4*, 6q1*, 6p2, 6q2, 7p1, 7p2*, 11p1*, 11q2*, 12p1, 12q2*, 13p1, and 19q1 (where the asterisk (*) denotes the major band associated with the chromosomal rearrangement). These early studies have implicated a role for chromosome 19 in OCa pathogenesis (Pejovic et al., 1992a; Taetle et al., 1999a,b; Thompson et al., 1994a, 1996). Improvements in molecular cytogenetic analyses by way of multi-colour karyotyping (Bayani and Squire, 2004a), readily revealed the cytogenetic complexity and heterogeneity of OCas (Bayani et al., 2002; Rao et al., 2002), further refining copy-number changes and preferential sites of chromosome breakage. Based on bulk high-throughput DNA analyses of OCas (derived largely from aCGH experiments), chromosome 19 has a relatively equal frequency of copy-number gain and loss of approximately 10% (reviewed by Gorringe and Campbell (2009)), with allelic imbalances showing more preferential gain in most of 19p and 19q (Gorringe et al., 2007). Very little information, however, regarding the nature, frequency and heterogeneity of structural alterations leading to local copy-number imbalances for chromosome 19 exists on a per cell basis. Moreover, deciphering the link between the observed global patterns of ploidy change and chromosomal instability with clinical factors, are becoming increasingly more relevant (Swanton et al., 2009). The chromosome 19 landscape was recently revisited by Micci et al. (2009) who undertook an extensive microdissection and reverse-FISH approach to identify the critical regions and loci on chromosome 19 that are subject to copy-number alterations in ovarian carcinomas; showing the frequent involvement of chromosome 19q13 in unbalanced translocations both intra- and inter-chromosomally. We have extended this observation to examine the 19q13 sub-bands: 19q13.2 and 19q13.3/4, demonstrating genomic instability and copy-number heterogeneity exists within this major band. As well, Tsao et al. (2001) virally immortalized human ovarian surface epithelial cells to identify early chromosomal events leading to tumorigenesis, and found that all immortalized cell lines showed gains of 19q13, and the whole gain of chromosome 19 prior to immoralization, re-enforcing the idea that chromosome 19 plays an important role in early

tumorigenesis and in maintaining tumorigenicity. A number of genes subject to copy-number changes, in addition to the *KLK* locus, have also been identified on 19q (Thompson et al., 1996), including *ACTN4* (Yamamoto et al., 2009), *AKT2* (Bellacosa et al., 1995; Nakayama et al., 2006) and *cyclin E* (Etemadmoghadam et al., 2009). The amplification of *AKT2* (19q13.2) has been demonstrated to be an important indicator of poor prognosis (Bellacosa et al., 1995) and implicated in the pathogenesis of high-grade ovarian cancers (Nakayama et al., 2007, 2006). Nine of our cases (11.1%) showed amplification of the 19q13.2 region, which we speculate may reflect *AKT2* amplification, since one of the BACs used for the 19q13.2 region (RP11-67A5), includes *AKT2*. Moreover, our observations in these chemotherapy naïve tumors indicate that these changes likely result from on-going tumorigenic processes rather than induced changes from radiation or chemotherapeutic agents.

Chromosome 19 is unique, possessing the greatest number of genes, gene families, CpG island density, and also contains a high-level of repeat elements despite being among the smallest chromosomes (Grimwood et al., 2004). Thus, it is not surprising that defects in DNA repair pathways associated with OCa (i.e. *BRCA1/2*) may be manifested in chromosomes with greater potential for damage or epigenetic modification when protective pathways have been compromised. Additionally, of the over 700 microRNAs (miRNAs) listed in the miRBase (http://www.mirbase.org), chromosome 19q also harbours the highest number of miRNAs, with greater than 60 of the approximately 90 miRNAs located on chromosome 19 mapping to 19q (specifically at 19q13.4). miRNAs, which have been shown to be important regulators of gene expression (reviewed by Barbarotto et al., 2008) and implicated in ovarian cancer (Nam et al., 2008; Zhang et al., 2006; Zhang et al., 2008), can potentially regulate hundreds of gene targets, thus the consequences of genomic changes at 19q can have far reaching implications than simply the expression of genes mapping to chromosome 19. In fact, the *KLK* findings in this study suggest that other mechanisms, in addition to copy-number, influence the observed overexpression of KLK6, which may include miRNAs. Recently, Chow et al. (2008) demonstrated *KLK6* and *KLK10* were regulated by miRNAs in a breast cancer cell line. Preliminary findings by our group (*Bayani et al. unpublished*) show that the expression of a number of miRNAs predicted to regulate *KLK6*, are decreased in ovarian cancer cell lines and primary tumors as compared to normal ovarian miRNAs. Certainly, the role of miRNAs to protein expression may explain the lack of correlation between *KLK* copy-number and KLK6 protein expression. Since the primary mechanism of gene regulation by miRNAs is mediated by the inhibition of translation, rather than the degradation of RNA transcript (Barbarotto et al., 2008); the observed increases in KLK6 RNA transcript influenced by copy-number and/or combinations of the aforementioned mechanisms, may not reflect the actual level of protein ultimately expressed. This could explain the observation for tumors with gains of the *KLK* locus and relatively moderate/low KLK6 IHC overexpression, wherein these tumor cells may continue to express those miRNAs regulating (and inhibiting) KLK6 translation. Similarly, for tumors with the observed net loss of the *KLK* locus and strong KLK6 protein expression, the miRNAs regulating KLK6 may be diminished in these cells, permitting translation of the up-regulated KLK6 transcript. Interestingly, a few of these miRNAs predicted to regulate *KLK6*, as well as other *KLK*s, are also located on chromosome 19q.

In summary we have demonstrated that the *KLK* locus at 19q13.3/4 is subject to high genomic instability and copy-number heterogeneity, mediated by structural rearrangements of 19q. Moreover, structural rearrangements on 19q are associated with tumor grade, and may be associated with, or a marker of the differential pathogenesis distinguishing low-grade and high-grade serous cancers. While *KLK6* copy-number, through the enumeration of the *KLK* locus, does not appear to directly regulate the observed KLK6 overexpression *per se*, it is one contributing factor. Lastly, the unique genomic properties of chromosome 19 suggest that the observed instability of 19q and the genes mapping to this location, including *KLK* gene members warrant further investigation.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgements

This work was funded by the Ontario Cancer Biomarker Network (OCBN). The authors wish to acknowledge Antoninus Soosaipillai for his technical advice.

REFERENCES

Al-Romaih, K., Bayani, J., Vorobyova, J., Karaskova, J., Park, P.C., Zielenska, M., Squire, J.A., 2003. Chromosomal instability in osteosarcoma and its association with centrosome abnormalities. Cancer Genet. Cytogenet. 144, 91–99.

Barbarotto, E., Schmittgen, T.D., Calin, G.A., 2008. Micrornas and cancer: profile, profile, profile. Int. J. Cancer 122, 969–977.

Bast Jr., R.C., Hennessy, B., Mills, G.B., 2009. The biology of ovarian cancer: new opportunities for translation. Nat. Rev. Cancer 9, 415–428.

Bayani, J., Brenton, J.D., Macgregor, P.F., Beheshti, B., Albert, M., Nallainathan, D., Karaskova, J., Rosen, B., Murphy, J., Laframboise, S., Zanke, B., Squire, J.A., 2002. Parallel analysis of sporadic primary ovarian carcinomas by spectral karyotyping, comparative genomic hybridization, and expression microarrays. Cancer Res. 62, 3466–3476.

Bayani, J., Paderova, J., Murphy, J., Rosen, B., Zielenska, M., Squire, J.A., 2008a. Distinct patterns of structural and numerical chromosomal instability characterize sporadic ovarian cancer. Neoplasia 10, 1057–1065.

Bayani, J., Paliouras, M., Planque, C., Shan, S.J., Graham, C., Squire, J.A., Diamandis, E.P., 2008b. Impact of cytogenetic and genomic aberrations of the kallikrein locus in ovarian cancer. Mol. Oncol. 2, 250–260.

Bayani, J., Selvarajah, S., Maire, G., Vukovic, B., Al-Romaih, K., Zielenska, M., Squire, J.A., 2007. Genomic mechanisms and measurement of structural and numerical instability in cancer cells. Semin. Cancer Biol. 17, 5–18.

Bayani, J., Squire, J., 2004a. Multi-color fish techniques. In: Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J., Yamada, K. (Eds.), Curr Protoc Cell Biol. John Wiley & Sons Inc., New York Unit 22.5.

Bayani, J., Squire, J.A., 2004b. Fluorescence in situ hybridization (FISH). In: Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J., Yamada, K. (Eds.), Curr Protoc Cell Biol. John Wiley & Sons Inc., New York Unit 22.4.

Bayani, J., Squire, J.A., 2004c. Preparation of cytogenetic specimens from tissue samples. In: Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J., Yamada, K. (Eds.), Curr Protoc Cell Biol. John Wiley & Sons Inc., New York Unit 22.2.

Bayani, J., Zielenska, M., Pandita, A., Al-Romaih, K., Karaskova, J., Harrison, K., Bridge, J.A., Sorensen, P., Thorner, P., Squire, J.A., 2003. Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. Genes Chromosomes Cancer 36, 7–16.

Bellacosa, A., de Feo, D., Godwin, A.K., Bell, D.W., Cheng, J.Q., Altomare, D.A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Benedetti Panici, P., Mancuso, S., Neri, G., Testa, J.R., 1995. Molecular alterations of the akt2 oncogene in ovarian and breast carcinomas. Int. J. Cancer 64, 280–285.

Blegen, H., Einhorn, N., Sjovall, K., Roschke, A., Ghadimi, B.M., McShane, L.M., Nilsson, B., Shah, K., Ried, T., Auer, G., 2000. Prognostic significance of cell cycle proteins and genomic instability in borderline, early and advanced stage ovarian carcinomas. Int. J. Gynecol. Cancer 10, 477–487.

Borgono, C.A., Diamandis, E.P., 2004. The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer 4, 876–890.

Borgono, C.A., Kishi, T., Scorilas, A., Harbeck, N., Dorn, J., Schmalfeldt, B., Schmitt, M., Diamandis, E.P., 2006. Human kallikrein 8 protein is a favorable prognostic marker in ovarian cancer. Clin. Cancer Res. 12, 1487–1493.

Ceccaroni, M., Chieco, P., Alboni, C., De Laco, P., Pagano, K., Ceccarelli, C., Santini, D., Taroni, B., Pelusi, G., 2004. P53 expression, DNA ploidy and mitotic index as prognostic factors in patients with epithelial ovarian carcinoma. Tumori 90, 600–606.

Chow, T.F., Crow, M., Earle, T., El-Said, H., Diamandis, E.P., Yousef, G.M., 2008. Kallikreins as microrna targets: an in silico and experimental-based analysis. Biol. Chem. 389, 731–738.

Day Jr., T.G., Gallager, H.S., Rutledge, F.N., 1975. Epithelial carcinoma of the ovary:Prognostic importance of histologic grade. Natl. Cancer Inst. Monogr. 42, 15–21.

Diamandis, E.P., Yousef, G.M., Soosaipillai, A.R., Grass, L., Porter, A., Little, S., Sotiropoulou, G., 2000. Immunofluorometric assay of human kallikrein 6 (zyme/protease m/neurosin) and preliminary clinical applications. Clin. Biochem. 33, 369–375.

Etemadmoghadam, D., deFazio, A., Beroukhim, R., Mermel, C., George, J., Getz, G., Tothill, R., Okamoto, A., Raeder, M.B., Harnett, P., Lade, S., Akslen, L.A., Tinker, A.V., Locandro, B., Alsop, K., Chiew, Y.E., Traficante, N., Fereday, S., Johnson, D., Fox, S., Sellers, W., Urashima, M., Salvesen, H.B., Meyerson, M., Bowtell, D., 2009. Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas. Clin. Cancer Res. 15, 1417–1427.

Frohling, S., Dohner, H., 2008. Chromosomal abnormalities in cancer. N. Engl. J. Med. 359, 722–734.

Fukasawa, K., 2005. Centrosome amplification, chromosome instability and cancer development. Cancer Lett. 230, 6–19.

Gorringe, K.L., Campbell, I.G., 2009. Large-scale genomic analysis of ovarian carcinomas. Mol. Oncol. 3, 157–164.

Gorringe, K.L., Chin, S.F., Pharoah, P., Staines, J.M., Oliveira, C., Edwards, P.A., Caldas, C., 2005. Evidence that both genetic instability and selection contribute to the accumulation of chromosome alterations in cancer. Carcinogenesis 26, 923–930.

Gorringe, K.L., Jacobs, S., Thompson, E.R., Sridhar, A., Qiu, W., Choong, D.Y., Campbell, I.G., 2007. High-resolution single nucleotide polymorphism array analysis of epithelial ovarian cancer reveals numerous microdeletions and amplifications. Clin. Cancer Res. 13, 4731–4739.

Grimwood, J., Gordon, L.A., Olsen, A., Terry, A., Schmutz, J., Lamerdin, J., Hellsten, U., Goodstein, D., Couronne, O., Tran-Gyamfi, M., Aerts, A., Altherr, M., Ashworth, L., Bajorek, E., Black, S., Branscomb, E., Caenepeel, S., Carrano, A., Caoile, C., Chan, Y.M., Christensen, M., Cleland, C.A., Copeland, A., Dalin, E., Dehal, P., Denys, M., Detter, J.C., Escobar, J., Flowers, D., Fotopulos, D., Garcia, C., Georgescu, A.M., Glavina, T., Gomez, M., Gonzales, E., Groza, M., Hammon, N., Hawkins, T., Haydu, L., Ho, I., Huang, W., Israni, S., Jett, J., Kadner, K., Kimball, H., Kobayashi, A., Larionov, V., Leem, S.H., Lopez, F., Lou, Y., Lowry, S., Malfatti, S., Martinez, D., McCready, P., Medina, C., Morgan, J., Nelson, K., Nolan, M., Ovcharenko, I., Pitluck, S., Pollard, M., Popkie, A.P., Predki, P., Quan, G., Ramirez, L., Rash, S., Retterer, J., Rodriguez, A., Rogers, S., Salamov, A., Salazar, A., She, X., Smith, D., Slezak, T., Solovyev, V., Thayer, N., Tice, H., Tsai, M., Ustaszewska, A., Vo, N., Wagner, M., Wheeler, J., Wu, K., Xie, G., Yang, J., Dubchak, I., Furey, T.S., DeJong, P., Dickson, M., Gordon, D., Eichler, E.E., Pennacchio, L.A., Richardson, P., Stubbs, L., Rokhsar, D.S., Myers, R.M., Rubin, E.M., Lucas, S.M., 2004. The DNA sequence and biology of human chromosome 19. Nature 428, 529–535.

Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.

Hoffman, B.R., Katsaros, D., Scorilas, A., Diamandis, P., Fracchioli, S., Rigault de la Longrais, I.A., Colgan, T., Puopolo, M., Giardina, G., Massobrio, M., Diamandis, E.P., 2002. Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br. J. Cancer 87, 763–771.

Inoue, Y., Yokobori, T., Yokoe, T., Toiyama, Y., Miki, C., Mimori, K., Mori, M., Kusunoki, M., 2010. Clinical significance of human kallikrein 7 gene expression in colorectal cancer. Ann. Surg. Oncol. Jun 11. [Epub ahead of print].

Kim, H., Scorilas, A., Katsaros, D., Yousef, G.M., Massobrio, M., Fracchioli, S., Piccinno, R., Gordini, G., Diamandis, E.P., 2001. Human kallikrein gene 5 (klk5) expression is an indicator of poor prognosis in ovarian cancer. Br. J. Cancer 84, 643–650.

Kountourakis, P., Psyrri, A., Scorilas, A., Camp, R., Markakis, S., Kowalski, D., Diamandis, E.P., Dimopoulos, M.A., 2008. Prognostic value of kallikrein-related peptidase 6 protein expression levels in advanced ovarian cancer evaluated by automated quantitative analysis (aqua). Cancer Sci. 99, 2224–2229.

Kuo, K.T., Guan, B., Feng, Y., Mao, T.L., Chen, X., Jinawath, N., Wang, Y., Kurman, R.J., Shih Ie, M., Wang, T.L., 2009. Analysis of DNA copy number alterations in ovarian serous tumors identifies new molecular genetic changes in low-grade and high-grade carcinomas. Cancer Res. 69, 4036–4042.

Kyriakopoulou, L.G., Yousef, G.M., Scorilas, A., Katsaros, D., Massobrio, M., Fracchioli, S., Diamandis, E.P., 2003. Prognostic value of quantitatively assessed klk7 expression in ovarian cancer. Clin. Biochem. 36, 135–143.

Lai, J., Myers, S.A., Lawrence, M.G., Odorico, D.M., Clements, J.A., 2009. Direct progesterone receptor and indirect androgen receptor interactions with the kallikrein-related peptidase 4 gene promoter in breast and prostate cancer. Mol. Cancer Res. 7, 129–141.

Lassus, H., Butzow, R., 2007. The classification of p53 immunohistochemical staining results and patient outcome in ovarian cancer. Br. J. Cancer 96, 1621–1622. Author Reply 1623–1624.

Lawrence, M.G., Lai, J., Clements, J.A., 2001. Kallikreins on steroids: Structure, function, and hormonal regulation of prostate-specific antigen and the extended kallikrein locus. Endocr. Rev. 4, 407–446.

Li, L., McCormack, A.A., Nicholson, J.M., Fabarius, A., Hehlmann, R., Sachs, R.K., Duesberg, P.H., 2009a. Cancer-causing karyotypes: chromosomal equilibria between destabilizing aneuploidy and stabilizing selection for oncogenic function. Cancer Genet. Cytogenet. 188, 1–25.

Li, X., Liu, J., Wang, Y., Zhang, L., Ning, L., Feng, Y., 2009b. Parallel underexpression of kallikrein 5 and kallikrein 7 mrna in breast malignancies. Cancer Sci. 100, 601–607.

Lilja, H., Ulmert, D., Vickers, A.J., 2008. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat. Rev. Cancer 8, 268–278.

Maire, G., Yoshimoto, M., Chilton-MacNeill, S., Thorner, P.S., Zielenska, M., Squire, J.A., 2009. Recurrent recql4 imbalance and increased gene expression levels are associated with structural chromosomal instability in sporadic osteosarcoma. Neoplasia 11, 260–268, 263 pp. following 268.

Mavridis, K., Scorilas, A., 2010. Prognostic value and biological role of the kallikrein-related peptidases in human malignancies. Future Oncol. 6, 269–285.

McManus, D.T., Murphy, M., Arthur, K., Hamilton, P.W., Russell, S.E., Toner, P.G., 1996. P53 mutation, allele loss on chromosome 17p, and DNA content in ovarian carcinoma. J. Pathol. 179, 177–182.

Micci, F., Weimer, J., Haugom, L., Skotheim, R.I., Grunewald, R., Abeler, V.M., Silins, I., Lothe, R.A., Trope, C.G., Arnold, N., Heim, S., 2009. Reverse painting of microdissected chromosome 19 markers in ovarian carcinoma identifies a complex rearrangement map. Genes Chromosomes Cancer 48, 184–193.

Nakayama, K., Nakayama, N., Jinawath, N., Salani, R., Kurman, R.J., Shih Ie, M., Wang, T.L., 2007. Amplicon profiles in ovarian serous carcinomas. Int. J. Cancer 120, 2613–2617.

Nakayama, K., Nakayama, N., Kurman, R.J., Cope, L., Pohl, G., Samuels, Y., Velculescu, V.E., Wang, T.L., Shih Ie, M., 2006. Sequence mutations and amplification of pik3ca and akt2 genes in purified ovarian serous neoplasms. Cancer Biol. Ther. 5, 779–785.

Nam, E.J., Yoon, H., Kim, S.W., Kim, H., Kim, Y.T., Kim, J.H., Kim, J.W., Kim, S., 2008. Microrna expression profiles in serous ovarian carcinoma. Clin. Cancer Res. 14, 2690–2695.

Nathalie, H.V., Chris, P., Serge, G., Catherine, C., Benjamin, B., Claire, B., Christelle, P., Briollais, L., Pascale, R., Marie-Lise, J., Yves, C., 2009. High kallikrein-related peptidase 6 in non-small cell lung cancer cells: an indicator of tumour proliferation and poor prognosis. J. Cell Mol. Med. 13, 4014–4022.

Ni, X., Zhang, W., Huang, K.C., Wang, Y., Ng, S.K., Mok, S.C., Berkowitz, R.S., Ng, S.W., 2004. Characterisation of human kallikrein 6/protease m expression in ovarian cancer. Br. J. Cancer 91, 725–731.

Obiezu, C.V., Scorilas, A., Katsaros, D., Massobrio, M., Yousef, G.M., Fracchioli, S., Rigault de la Longrais, I.A., Arisio, R., Diamandis, E.P., 2001. Higher human kallikrein gene 4 (klk4) expression indicates poor prognosis of ovarian cancer patients. Clin. Cancer Res. 7, 2380–2386.

Pampalakis, G., Arampatzidou, M., Amoutzias, G., Kossida, S., Sotiropoulou, G., 2008. Identification and analysis of mammalian klk6 orthologue genes for prediction of physiological substrates. Comput. Biol. Chem. 32, 111–121.

Pampalakis, G., Prosnikli, E., Agalioti, T., Vlahou, A., Zoumpourlis, V., Sotiropoulou, G., 2009. A tumor-protective role for human kallikrein-related peptidase 6 in breast cancer mediated by inhibition of epithelial-to-mesenchymal transition. Cancer Res. 69, 3779–3787.

Pampalakis, G., Sotiropoulou, G., 2006. Multiple mechanisms underlie the aberrant expression of the human kallikrein 6 gene in breast cancer. Biol. Chem. 387, 773–782.

Pejovic, T., Heim, S., Mandahl, N., Baldetorp, B., Elmfors, B., Floderus, U.M., Furgyik, S., Helm, G., Himmelmann, A., Willen, H., et al., 1992a. Chromosome aberrations in 35 primary ovarian carcinomas. Genes Chromosomes Cancer 4, 58–68.

Pejovic, T., Himmelmann, A., Heim, S., Mandahl, N., Floderus, U.M., Furgyik, S., Elmfors, B., Helm, G., Willen, H., Mitelman, F., 1992b. Prognostic impact of chromosome aberrations in ovarian cancer. Br. J. Cancer 65, 282–286.

Petraki, C.D., Karavana, V.N., Skoufogiannis, P.T., Little, S.P., Howarth, D.J., Yousef, G.M., Diamandis, E.P., 2001. The spectrum of human kallikrein 6 (zyme/protease m/neurosin) expression in human tissues as assessed by immunohistochemistry. J. Histochem. Cytochem. 49, 1431–1441.

Petraki, C.D., Papanastasiou, P.A., Karavana, V.N., Diamandis, E.P., 2006. Cellular distribution of human tissue kallikreins: immunohistochemical localization. Biol. Chem. 387, 653–663.

Pettus, J.R., Johnson, J.J., Shi, Z., Davis, J.W., Koblinski, J., Ghosh, S., Liu, Y., Ravosa, M.J., Frazier, S., Stack, M.S., 2009. Multiple kallikrein (klk 5, 7, 8, and 10) expression in squamous cell carcinoma of the oral cavity. Histol. Histopathol. 24, 197–207.

Planque, C., de Monte, M., Guyetant, S., Rollin, J., Desmazes, C., Panel, V., Lemarie, E., Courty, Y., 2005. Klk5 and klk7, two members of the human tissue kallikrein family, are differentially expressed in lung cancer. Biochem. Biophys. Res. Commun. 329, 1260–1266.

Rao, P.H., Harris, C.P., Yan Lu, X., Li, X.N., Mok, S.C., Lau, C.C., 2002. Multicolor spectral karyotyping of serous ovarian adenocarcinoma. Genes Chromosomes Cancer 33, 123–132.

Ricciardelli, C., Oehler, M.K., 2009. Diverse molecular pathways in ovarian cancer and their clinical significance. Maturitas 62, 270–275.

Sadikovic, B., Al-Romaih, K., Squire, J.A., Zielenska, M., 2008. Cause and consequences of genetic and epigenetic alterations in human cancer. Curr. Genomics 9, 394–408.

Shan, S.J., Scorilas, A., Katsaros, D., Diamandis, E.P., 2007. Transcriptional upregulation of human tissue kallikrein 6 in ovarian cancer: clinical and mechanistic aspects. Br. J. Cancer 96, 362–372.

Shan, S.J., Scorilas, A., Katsaros, D., Rigault de la Longrais, I., Massobrio, M., Diamandis, E.P., 2006. Unfavorable prognostic value of human kallikrein 7 quantified by elisa in ovarian cancer cytosols. Clin. Chem. 52, 1879–1886.

Shaw, J.L., Diamandis, E.P., 2008. Regulation of human tissue kallikrein-related peptidase expression by steroid hormones in 32 cell lines. Biol. Chem. 389, 1409–1419.

Shih Ie, M., Kurman, R.J., 2004. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am. J. Pathol. 164, 1511–1518.

Shinoda, Y., Kozaki, K., Imoto, I., Obara, W., Tsuda, H., Mizutani, Y., Shuin, T., Fujioka, T., Miki, T., Inazawa, J., 2007. Association of klk5 overexpression with invasiveness of urinary bladder carcinoma cells. Cancer Sci. 98, 1078–1086.

Shridhar, V., Lee, J., Pandita, A., Iturria, S., Avula, R., Staub, J., Morrissey, M., Calhoun, E., Sen, A., Kalli, K., Keeney, G., Roche, P., Cliby, W., Lu, K., Schmandt, R., Mills, G.B., Bast Jr., R.C., James, C.D., Couch, F.J., Hartmann, L.C., Lillie, J., Smith, D.I., 2001. Genetic analysis of early- versus late-stage ovarian tumors. Cancer Res. 61, 5895–5904.

Sidiropoulos, M., Pampalakis, G., Sotiropoulou, G., Katsaros, D., Diamandis, E.P., 2005. Downregulation of human kallikrein 10 (klk10/nes1) by cpg island hypermethylation in breast, ovarian and prostate cancers. Tumour Biol. 26, 324–336.

Swanton, C., Nicke, B., Schuett, M., Eklund, A.C., Ng, C., Li, Q., Hardcastle, T., Lee, A., Roy, R., East, P., Kschischo, M., Endesfelder, D., Wylie, P., Kim, S.N., Chen, J.G., Howell, M., Ried, T., Habermann, J.K., Auer, G., Brenton, J.D., Szallasi, Z.,

Downward, J., 2009. Chromosomal instability determines taxane response. Proc. Natl. Acad. Sci. USA 106, 8671–8676.
Taetle, R., Aickin, M., Panda, L., Emerson, J., Roe, D., Thompson, F., Davis, J., Trent, J., Alberts, D., 1999a. Chromosome abnormalities in ovarian adenocarcinoma: Ii. Prognostic impact of nonrandom chromosome abnormalities in 244 cases. Genes Chromosomes Cancer 25, 46–52.
Taetle, R., Aickin, M., Yang, J.M., Panda, L., Emerson, J., Roe, D., Adair, L., Thompson, F., Liu, Y., Wisner, L., Davis, J.R., Trent, J., Alberts, D.S., 1999b. Chromosome abnormalities in ovarian adenocarcinoma: I. Nonrandom chromosome abnormalities from 244 cases. Genes Chromosomes Cancer 25, 290–300.
Thompson, F.H., Emerson, J., Alberts, D., Liu, Y., Guan, X.Y., Burgess, A., Fox, S., Taetle, R., Weinstein, R., Makar, R., et al., 1994a. Clonal chromosome abnormalities in 54 cases of ovarian carcinoma. Cancer Genet. Cytogenet. 73, 33–45.
Thompson, F.H., Liu, Y., Emerson, J., Weinstein, R., Makar, R., Trent, J.M., Taetle, R., Alberts, D.S., 1994b. Simple numeric abnormalities as primary karyotype changes in ovarian carcinoma. Genes Chromosomes Cancer 10, 262–266.
Thompson, F.H., Nelson, M.A., Trent, J.M., Guan, X.Y., Liu, Y., Yang, J.M., Emerson, J., Adair, L., Wymer, J., Balfour, C., Massey, K., Weinstein, R., Alberts, D.S., Taetle, R., 1996. Amplification of 19q13.1-q13.2 sequences in ovarian cancer. G-band, fish, and molecular studies. Cancer Genet. Cytogenet. 87, 55–62.
Tomasini, R., Mak, T.W., Melino, G., 2008. The impact of p53 and p73 on aneuploidy and cancer. Trends Cell Biol. 18, 244–252.
Tsao, S.W., Wong, N., Wang, X., Liu, Y., Wan, T.S., Fung, L.F., Lancaster, W.D., Gregoire, L., Wong, Y.C., 2001. Nonrandom chromosomal imbalances in human ovarian surface epithelial cells immortalized by hpv16-e6e7 viral oncogenes. Cancer Genet. Cytogenet. 130, 141–149.
Vojtesek, B., Bartek, J., Midgley, C.A., Lane, D.P., 1992. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. J. Immunol. Methods 151, 237–244.
Vukovic, B., Beheshti, B., Park, P., Lim, G., Bayani, J., Zielenska, M., Squire, J.A., 2007. Correlating breakage-fusion-bridge events with the overall chromosomal instability and in vitro karyotype evolution in prostate cancer. Cytogenet. Genome Res. 116, 1–11.
White, N.M., Bui, A., Mejia-Guerrero, S., Chao, J., Soosaipillai, A., Youssef, Y., Mankaruos, M., Honey, R.J., Stewart, R., Pace, K.T., Sugar, L., Diamandis, E.P., Dore, J., Yousef, G.M., 2010. Dysregulation of kallikrein-related peptidases in renal cell carcinoma: potential targets of mirnas. Biol. Chem. 391, 411–423.
White, N.M., Mathews, M., Yousef, G.M., Prizada, A., Popadiuk, C., Dore, J.J., 2009. Klk6 and klk13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer 7, 1107–1113.
Yamamoto, S., Tsuda, H., Honda, K., Onozato, K., Takano, M., Tamai, S., Imoto, I., Inazawa, J., Yamada, T., Matsubara, O., 2009. Actinin-4 gene amplification in ovarian cancer: a candidate oncogene associated with poor patient prognosis and tumor chemoresistance. Mod. Pathol. 22, 499–507.
Yousef, G.M., Chang, A., Scorilas, A., Diamandis, E.P., 2000. Genomic organization of the human kallikrein gene family on chromosome 19q13.3-q13.4. Biochem. Biophys. Res. Commun. 276, 125–133.
Yousef, G.M., Polymeris, M.E., Grass, L., Soosaipillai, A., Chan, P.C., Scorilas, A., Borgono, C., Harbeck, N., Schmalfeldt, B., Dorn, J., Schmitt, M., Diamandis, E.P., 2003a. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res. 63, 3958–3965.
Yousef, G.M., Polymeris, M.E., Yacoub, G.M., Scorilas, A., Soosaipillai, A., Popalis, C., Fracchioli, S., Katsaros, D., Diamandis, E.P., 2003b. Parallel overexpression of seven kallikrein genes in ovarian cancer. Cancer Res. 63, 2223–2227.
Zhang, L., Huang, J., Yang, N., Greshock, J., Megraw, M.S., Giannakakis, A., Liang, S., Naylor, T.L., Barchetti, A., Ward, M.R., Yao, G., Medina, A., O'Brien-Jenkins, A., Katsaros, D., Hatzigeorgiou, A., Gimotty, P.A., Weber, B.L., Coukos, G., 2006. Micrornas exhibit high frequency genomic alterations in human cancer. Proc. Natl. Acad. Sci. USA 103, 9136–9141.
Zhang, L., Volinia, S., Bonome, T., Calin, G.A., Greshock, J., Yang, N., Liu, C.G., Giannakakis, A., Alexiou, P., Hasegawa, K., Johnstone, C.N., Megraw, M.S., Adams, S., Lassus, H., Huang, J., Kaur, S., Liang, S., Sethupathy, P., Leminen, A., Simossis, V.A., Sandaltzopoulos, R., Naomoto, Y., Katsaros, D., Gimotty, P.A., DeMichele, A., Huang, Q., Butzow, R., Rustgi, A.K., Weber, B.L., Birrer, M.J., Hatzigeorgiou, A.G., Croce, C.M., Coukos, G., 2008. Genomic and epigenetic alterations deregulate microrna expression in human epithelial ovarian cancer. Proc. Natl. Acad. Sci. USA 105, 7004–7009.